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Cloning of a Mineral Phosphate-Solubilizing Gene from *Pseudomonas cepacia*

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We have recently shown that the ability of some gram-negative bacteria to dissolve poorly soluble calcium phosphates (Mps^+ phenotype) is the result of periplasmic oxidation of glucose to gluconic acid via the quinoprotein glucose dehydrogenase (GDH), a component of the direct oxidation pathway. *Escherichia coli* K-12 derivatives synthesize apo-GDH but not the cofactor pyrroloquinoline-quinone (PQQ) essential for formation of the holoenzyme. Therefore, in the absence of exogenous PQQ, these strains do not produce gluconic acid and are Mps^-. Evidence is presented to show that expression of a single 396-base *Pseudomonas cepacia* open reading frame (designated *gabY*) in *E. coli* JM109 (a K-12 derivative) was sufficient to induce the Mps^+ phenotype and production of gluconic acid. We present the nucleotide sequence of this open reading frame which coded for a protein (GabY) with a deduced M_r of 14,235. Coupled transcription-translation of a plasmid (pSLY4 or pGAB1) carrying *gabY* resulted in production of a protein with an M_r of 14,750. Disruption of the open reading frame of *gabY* via site-directed mutagenesis changed the phenotype to Mps^- and eliminated gluconic acid production. The deduced amino acid sequence of *gabY* has no apparent homology with those of previously cloned direct oxidation pathway genes but does share regions highly homologous with the histidine permease system membrane-bound protein HisQ as well as other proteins in this family. In the presence of 1 μM exogenous PQQ, both JM109(pSLY4) and JM109(pGAB1) produced 10 times as much gluconic acid as was seen with either the plasmid or exogenous PQQ alone. The presence of pGAB1 was also sufficient to cause production of gluconic acid in *E. coli* HB101 (a K-12-B hybrid). In AG121, an apoGDH$^-$, Tn*5* mutant of HB101, the presence of pGAB1 did not cause the production of gluconic acid.

It has long been known that some gram-negative bacteria have the ability to dissolve poorly soluble calcium phosphates such as hydroxyapatite and rock phosphate ore. We have named this phenotype the mineral phosphate-solubilizing (Mps^+) phenotype. We are interested in identification and characterization of functional Mps genes (6–9). The Mps phenotype has been of interest to agricultural microbiologists and microbial ecologists for much of this century because the action of Mps^+ bacteria on poorly soluble calcium phosphates in soils can enhance the availability of P_i for microbial and/or plant growth (6, 7). The Mps phenotype has historically been associated with the production of organic acids. Recently, we have shown that the strong Mps^+ phenotype exhibited by *Erwinia herbicola* and *Pseudomonas cepacia* is the result of gluconic acid-mediated dissolution of the calcium phosphate (9). This gluconic acid is produced in the periplasmic space by the direct oxidation pathway. The first step in this pathway is the oxidation of glucose to gluconic acid via the membrane-bound glucose dehydrogenase (GDH; EC 1.1.99.17). This enzyme contains 2,7,9-tricarboxyl-1H-pyrrolo[2,3-f]-quinoline-4,5-dione (pyrroloquinoline-quinone [PQQ]) as a prosthetic group, so that the functional holoenzyme is a quinoprotein (1, 4).

GDH is a member of the largest group of quinoproteins, those that use the cofactor PQQ (4, 5). Quinoproteins play a major role in the regulation of bioenergetic processes in many gram-negative bacteria including *Pseudomonas* species (4). For many species of *Pseudomonas*, the nonphosphorylating oxidation pathway is a primary pathway for aldose sugar utilization (16). The enzymes of the direct oxidation pathway are oriented in the cytoplasmic membrane such that glucose (or other aldose sugars) undergoes up to three oxidations of two electrons and two protons in the periplasmic space. As a result of these oxidations, gluconic, 2-ketogluconic, and/or 2,5-diketogluconic acid is formed in the periplasmic space, with the resultant acidification of this region and, ultimately, the adjacent medium as well (9).

We are interested in using this mineral phosphate-solubilizing phenotype as the basis for a renewable phosphate fertilizer technology for crop growth (6, 8, 9). In addition, we are beginning to ask questions about a possible ecophysiological role for Mps (i.e., direct oxidation pathway or other) genes in rhizobacteria isolated from plants growing in P_i-limited environments (7). Our ability to identify Mps genes in gram-negative bacteria has resulted from studies in which genes from Mps^+ bacteria have been expressed in *Escherichia coli. E. coli* is Mps^- because it does not synthesize holo-GDH and is therefore incapable of initiating the direct oxidation pathway. *E. coli* K-12 has been shown to constitutively synthesize apo-GDH but not PQQ. It was postulated that PQQ was absorbed from the environment as a vitamin (10). The current model is that apo-GDH is integrated into its location on the outer face of the cytoplasmic membrane. When cofactor PQQ is present in the medium, it binds to apo-GDH to form the holoenzyme. If functional PQQ synthesis genes from another bacterium are supplied, GDH activity is seen (10, 17). It is generally believed that *E. coli* K-12 (as well as other strains) lacks genes for PQQ

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
P. cepacia E-37	Wild type	Rogers et al.
E. coli JM109	F^- *hsdS20* (r_B^- m_B^-) *supE44 recA13 ara14 proA2 rpsL20*(Str^r) *xyl-5 mtl-q1 galK2 lacY1* λ^-	Stratagene, Inc.
pGEM4Z	Promega cloning vector pGEM4Z	Promega, Inc.
pSLY4	pGEM4Z containing a 2.6-kb insert of E-37 DNA	This study
pGAB1	Subclone of pSLY4 containing 0.7 kb of the original 2.6-kb insert 3′ to the SP6 promoter of pGEM4Z that includes the intact ORF	This study
pGAB1d	pGAB1 modified by Klenow fragment repair at the unique *Xho*I site of the insert to disrupt the ORF of *gabY*	This study
pGP478	*Eco*RI-*Sal*I fragment of *E. coli* DNA cloned into pBR322, containing *gcd* under control of its own promoter	Nora Goosen laboratory

biosynthesis, although one report of a mutant that apparently expresses PQQ synthase genes has been published (2).

In this paper, we report the construction of a plasmid containing a functional mineral phosphate-solubilizing gene from *P. cepacia* E-37. The expression of this gene on a plasmid in *E. coli* JM109 or HB101 resulted in both dissolution of exogenous hydroxyapatite and production of gluconic acid. This gene (designated *gabY*) contained only one apparent open reading frame (ORF). The complete nucleotide sequence of *gabY* is reported. The deduced amino acid sequence of *gabY* coded for a protein with a molecular mass of 14.235 kDa (GabY), which agreed well with coupled transcription-translation data showing the production of a 14.75-kDa translation product. Disruption of *gabY* via site-directed mutagenesis resulted in the loss of both the Mps^+ phenotype and the ability to produce gluconic acid. GabY has no apparent homology with previously cloned putative PQQ synthase genes but does share regions highly homologous with the histidine permease system membrane-bound protein HisQ (14) as well as other proteins in this family.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* JM109, HB101, and AG121 and plasmid-carrying derivatives were cultured in LB broth (21) or defined minimal medium (as described below) at 37°C. *P. cepacia* E-37, a wild-type strain originally isolated by Rogers et al. (18), was cultured in LB broth at 28°C. E-37 was resistant to several antibiotics and was routinely cultured with 60 μg of streptomycin per ml and 20 μg of ampicillin per ml. Plasmid selection was conducted with ampicillin at a concentration of 100 μg/ml.

Construction of the plasmid library and subcloning. Restriction enzymes, calf intestinal phosphatase, T4 DNA ligase, and *E. coli* DNA polymerase I (Klenow fragment) were purchased from Promega and were used according to the manufacturer's recommendations and/or according to the methods described by Sambrook et al. (21). A genomic plasmid library of E-37 DNA was constructed by the methods described by Sambrook et al. (21). Total DNA was isolated from E-37 and purified on a cesium chloride gradient. This DNA was partially restricted with *Sau*3A, and the 5- to 15-kb fraction was isolated from low-melting-point agarose. This 5- to 15-kb fraction was ligated into the *Bam*HI site of the plasmid vector pGEM4Z (Promega), and the ligation product was transformed into competent *E. coli* JM109. Approximately 1,000 clones were screened for the Mps^+ phenotype (i.e., dissolution of hydroxyapatite) on M9-HAP agar as previously described (8, 17). The clone pSLY4 was selected on the basis of exhibition of the Mps^+ phenotype (see Fig. 6). Plasmids were isolated via minipreps, and preliminary restriction analyses were conducted according to the method of Sambrook et al. (21).

Primer walking from both ends of the pSLY4 insert allowed us to identify possible ORFs. However, compression problems stopped the complete sequencing of the entire ~2.4-kb insert. The sequencing data indicated a candidate ORF beginning 226 bases downstream from the SP6 promoter that coded for a polypeptide with a deduced M_r similar to that of the major coupled transcription-translation product of pSLY4. We then used Promega's Erase-A-Base system to create subclones of pSLY4 containing progressive unidirectional deletions that removed much of the DNA downstream from this ORF (Fig. 1). A progressive series of deletion mutants missing approximately 300, 600, 900, 1,200, 1,500, and 1,800 bases all retained Mps^+ activity in plate assays. The smallest insert contained approximately 700 bases of *P. cepacia* DNA (Fig. 1). This plasmid, pGAB1, was used for both coupled transcription-translation and gluconic acid production studies.

In vitro transcription-translation and analysis of plasmid-encoded proteins. Plasmids pSLY4 and pGAB1 were analyzed along with appropriate controls by using the coupled transcription-translation system purchased from Amersham Inc. according to the manufacturer's protocol. The translation reaction mixture contained 10 μCi of [^{35}S]methionine. Translation products were initially separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using the system described by Laemmli (15). Translation products were visualized via autoradiography after treatment with En^{3}Hance (New England Nuclear). Identification of low-molecular-mass translation products prompted further separations with the Tricine-based SDS-PAGE system of Schagger and von Jagow (22).

DNA sequencing. Supercoiled plasmids were sequenced according to the chain termination method of Sanger et al. (20). Initially, DNA was sequenced with the T7 and SP6 primers homologous to the T7 and SP6 promoter sites flanking the multiple cloning site of pGEM4Z. Sequences were extended on both the coding and template strands by iterative synthesis of primers homologous to the deduced downstream end of the strand being sequenced (primer walking). Compression problems limited sequencing to the first ~800 bases downstream from SP6 and the first ~500 bases upstream from T7 (see Fig. 1 for orientation). Therefore, only about one-half of the pSLY4 insert was sequenced.

Site-directed mutagenesis. In order to further demonstrate that the major ORF of pGAB1 was responsible for the Mps^+ phenotype, we performed site-directed mutagenesis at the unique *Xho*I site within the ORF. pGAB1 was restricted with *Xho*I prior to blunt ending with Klenow enzyme. The blunt ends were ligated to obtain the *Xho*I site-disrupted insert of pGAB1d.

Determination of organic acid concentrations during bacterial growth. Liquid medium (GMS medium) consisted of (for 1 liter) 10 g of glucose, 2 g of $(NH_4)_2SO_4$, 0.3 g of $MgCl_2 \cdot 6H_2O$, 0.001 g of $MnCl_2 \cdot 4H_2O$, 0.0006 g of $FeSO_4 \cdot 7H_2O$, and 0.0006 g of $NaMoO_4$. Amino acids or thiamine (20 μg/ml), ampicillin (100 μg/ml), and tricalcium phosphate (20 mg/ml) were added. Fifty

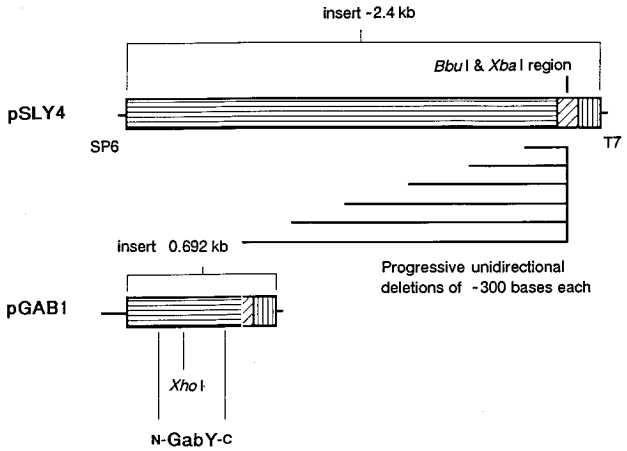

FIG. 1. Generalized subcloning strategy for generation of progressive unidirectional deletions of pSLY4. Deletions began immediately upstream from the T7 promoter of the original pGEM4Z cloning vector and moved upstream towards the SP6 end. The subclone with the largest deletion was designated pGAB1.

TABLE 2. Gluconic acid production by *E. coli* strains[a]

Strain or medium	Exogenous PQQ	Gluconic acid concn in medium (mM)
HB101	+	1.5
HB101(pGAB1)	+	32.0
AG121	+	Not detected
AG121(pGAB1)	+	Not detected
AG121(pGP478)	+	7.2
HB101	−	Not detected
HB101(pGAB1)	−	3.0
AG121	−	Not detected
AG121(pGAB1)	−	Not detected
AG121(pGP478)	−	Not detected
Medium	+ or −	Not detected
Medium + 6 mM D-gluconic acid	+ or −	6.0

[a] The detection limit of the enzyme assay was ~0.25 mM. +, exogenous PQQ added; −, no exogenous PQQ.

milliliters of medium was inoculated with 5 ml of 48-h-old (stationary phase) liquid culture of the various bacteria. After inoculation, the flasks were placed on a shaker and the bacteria were grown at 37°C for 24 h. The supernatant of each culture was obtained by centrifugation at 10,000 rpm (SS-34 rotor) for 10 min.

For the experiment to determine gluconic acid levels (see Fig. 4), organic acids were separated by reverse-phase, ion-pairing high-performance liquid chromatography (C_{18} column). The mobile phase consisted of 50 mM sodium phosphate and 5 mM tetrabutylammonium hydrogen sulfate, pH 6.5 (95%), plus acetonitrile (5%). The flow rate was 0.25 ml/min. Detection was performed via A_{220}. The identification of unknown organic acids was based on comparison of the elution time of the unknown acid with the elution times of multiple standards. The detection limit for gluconic acid in culture supernatant was approximately 0.1 mM. The production of gluconic acid by JM109(pSLY4) and JM109(pGAB1) was confirmed by coupled enzyme assay as described below.

For the studies involving the Tn*5* knockout of apo-GDH activity, gluconic acid concentration was determined with the Boehringer Mannheim gluconic acid detection kit (product 428 191). This system uses gluconate kinase and 6-phosphogluconate dehydrogenase to generate NADPH stoichiometric with the amount of D-gluconate in solution. The increase in NADPH is measured by means of A_{340}. *E. coli* HB101 was used in this study because of the availability of an apoGDH$^-$ strain, AG121, previously constructed in our laboratory. AG121 was isolated via lambda Tn*5* mutagenesis of HB101.

Lambda Tn*5* mutagenesis. Lambda Tn*5* was obtained from the laboratory of S.-T. Liu (Chang-Gung Medical College, Tainan, Taiwan). Lambda Tn*5* mutagenesis of *E. coli* HB101 was carried out as described by Ruvkin and Ausubel (19) with slight modifications. Tn*5* mutants were picked from kanamycin plates and screened on HAP medium for the Mps$^-$ phenotype as described above. The presence of Tn*5* was confirmed by Southern hybridization. The Mps$^-$ phenotype was attributed to a functional knockout of apo-GDH because no gluconic acid production was detected in the presence of exogenous PQQ, whereas HB101 produced gluconic acid in the presence of exogenous PQQ (Table 2). As a further control, the cloned *E. coli* apo-GDH gene, *gcd*, under control of its own promoter on the plasmid pGP478 was obtained from Nora Goosen (3). pGP478 was transformed into both HB101 and AG121 by standard methods (21).

Nucleotide sequence accession number. The GenBank accession number of the *P. cepacia* E-37 ORF designated *gabY* is U10242 (Fig. 2).

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        10         20         30         40         50         60         70
CCTCGCTGGCACCTGGCAAACCGTCAAGCTGGCGGTGCTTTCGCTTGCGCTGTCGTTCCTGCTGGGCCTG

        80         90        100        110        120        130        140
CTCGGCGCGGGCCGAACGTGTCGCAACCGGTTCACAAACGGCGTCGGCACCGTCTACACGACGCTGATCC

       150        160        170        180        190        200        209
GCGGCGTGCCCGACCTCGTGCTCACCGCTGCTGTTCTACAGCCTGCAGATCTGGCTGAACATGGCGACC
                                                              METAlaThr

      218        227        236        245        254        263        272
GACGCACTCGGCTGGGACCAGATCGACATCGACCCGTTCCTCGCCGGCGTGCTCGTGCTCGGCTTCATC
AspAlaLeuGlyTrpAspGlnIleAspIleAspProPheLeuAlaGlyValLeuValLeuGlyPheIle

281        290        299        308        317        326        335        344
TACGGCGCGTACTTCACCGAGACGTTCCGCGGCGGTTCCTGTCGGTGCCGCGCGGCCAGCTCGAGGCCG
TyrGlyAlaTyrPheThrGluThrPheArgGlyGlySerCysArgCysArgAlaAlaSerSerArgPro

   353        362        371        380        389        398        407        416
GCAGCGGTACGGGATGACCAGCTGGCAGGTGTTCACGCGGATCATGTTCCCGCAGATGATGCGCTTCGC
AlaAlaValArgAspAspGluLeuAlaGlyValHisAlaAspHisValProAlaAspAspAlaLeuArg

      425        434        443        452        461        470        479
GCCGGGCATCGCAACAACTGGCAGGTGCTCGTGAAGTCTACCGCGCTCGTGTCGATCATCGGGCTCGCC
AlaGlyHisArgAsnAsnTrpGlnValLeuValLysSerThrAlaLeuValSerIleIleGlyLeuAla

488        497        506        515        524        533        542        551
GACGTAGTGAAGGCGTCGCAGGATGCCGGCAAGGGCACGCTGCGGTTCTTCTTCTTCACGCTGCTCGCG
AspValValLysAlaSerGlnAspAlaGlyLysGlyThrLeuArgPhePhePhePheThrLeuLeuAla

   560        569        578        587        596        609        619
GGACCGTCTACCTCGCGATCACGACGATCTCGAACTTCGTACTGATGTGGCTCGAAAAGCGCTACTCGA
GlyProSerThrSerArgSerArgArgSerArgThrSerTyr .

    629        639        649        659        669        679        689
CCGGCGTCCACAAGCTGACCTCTGATCGAACTCATCCAAGAATACTGGCACAACTATCTCTTCTCCGAC
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FIG. 2. Nucleotide sequence of the insert of pGAB1. Translation of the putative ORF of *gabY* is indicated below the sequence, and nucleotide position numbers are indicated above the sequence.

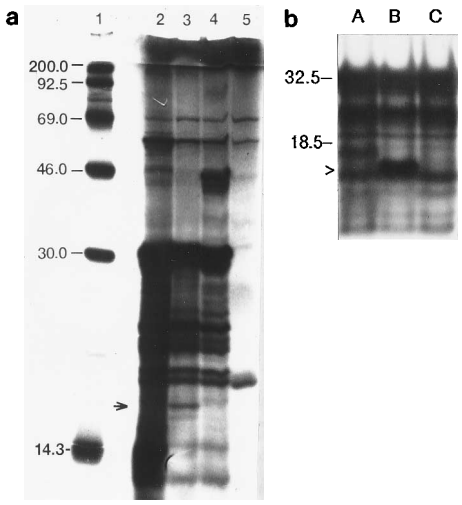

FIG. 3. (a) Autoradiogram showing apparent production of GabY by pSLY4 in a coupled in vitro transcription-translation system. Translation products were separated by SDS-PAGE using a linear 6 to 16% acrylamide gradient Tricine gel (1.0 mm by 20.0 cm) by a procedure modified from that of Schagger and von Jagow (20). Lane 1, molecular weight markers; lane 2, pGEM4Z; lane 3, pSLY4; lane 4, pSLY1 (an independent selection from the *P. cepacia* library with no apparent homology to the insert of pSLY4); lane 5, blank (no added DNA). A 14.7-kDa translation product present only in the pSLY4 sample is indicated by the arrow. Shorter exposure times revealed no 14.7-kDa translation product in the pGEM4Z lane (data not shown). (b) Autoradiogram showing apparent production of GabY by pGAB1 but not by pGEM4Z or pGAB1d in a coupled in vitro transcription-translation system. Translation products were separated by SDS-PAGE using a 10% acrylamide (3% stack) Tricine gel (0.75 mm by 7.0 cm) by a procedure modified from that of Schagger and von Jagow (20). Lane A, pGEM4Z; lane B, pGAB1; lane C, pGAB1d. The position of GabY is indicated by the caret. Sizes (in kilodaltons) are indicated on the left of each gel.

RESULTS AND DISCUSSION

We are interested in the genetic and biochemical bases of high-efficiency calcium phosphate solubilization by gram-negative bacteria (6–9, 17). Towards this end, we have developed a strategy for the isolation of Mps genes based on the fact that, in plate assays, *E. coli* cannot visibly solubilize poorly soluble mineral phosphates whereas *E. herbicola*, *P. cepacia* and other Mps$^+$ bacteria can (6, 8). We have previously shown that a recombinant *E. coli* strain carrying a plasmid containing *E. herbicola* DNA exhibited both the Mps$^+$ phenotype and gluconic acid production (17). DNA sequence analysis of the *E. herbicola* DNA indicated probable involvement in PQQ biosynthesis resulting in the presence of holo-GDH. The plasmid pSLY4 was identified by a similar screening technique (see Fig. 6).

DNA sequencing from both the SP6 and T7 ends of the insert of pSLY4 resulted in the identification of only one apparent ORF of sufficient length to code for a protein with a deduced molecular mass similar to that of the observed tran-

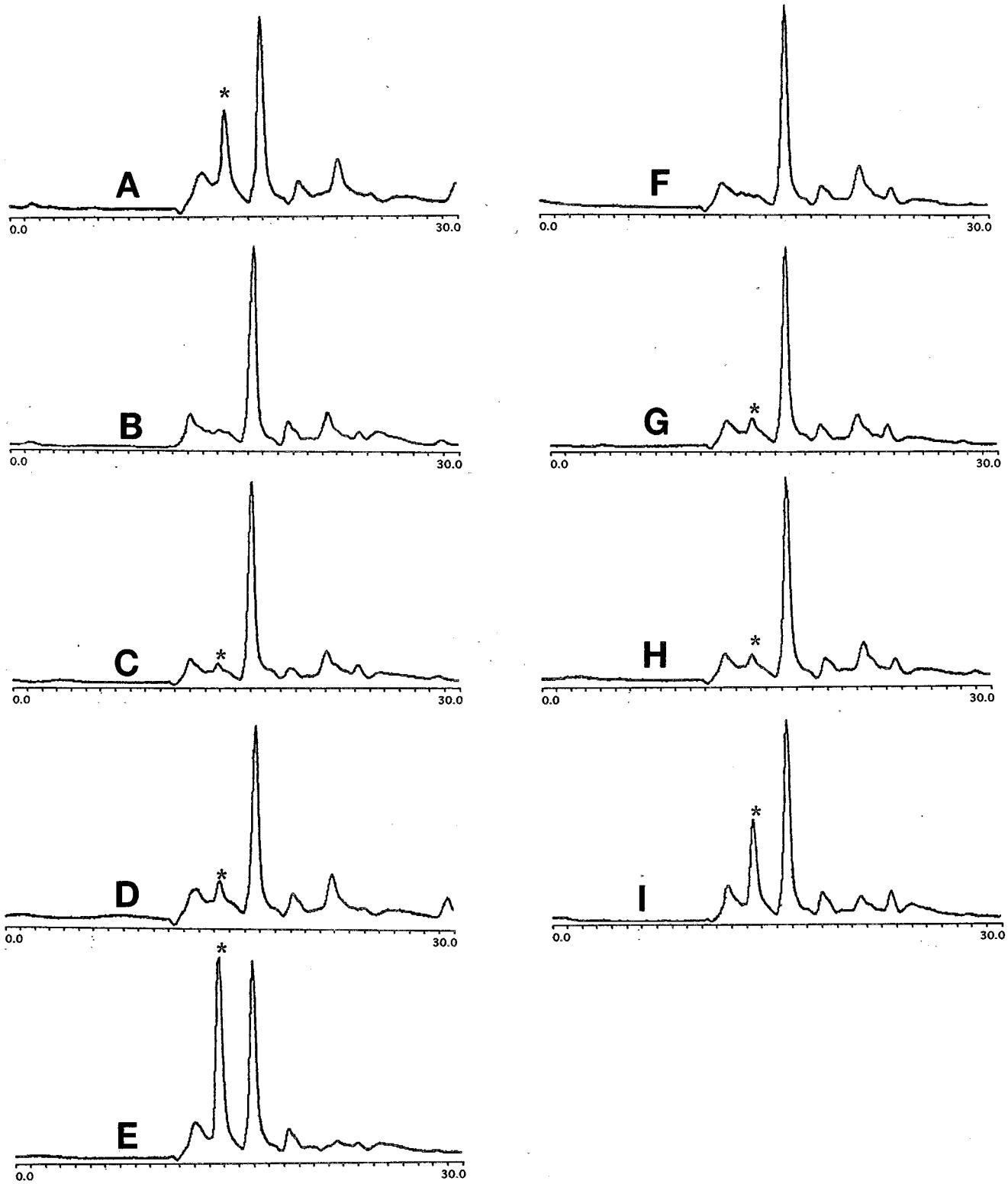

FIG. 4. Determination of gluconic acid in the supernatant of JM109 cells carrying various plasmids. The chromatographic separations show A_{220} (*y* axis) versus time (minutes). The concentrations used were as follows: with no plasmid, 2 mM exogenous gluconic acid (A); with pGEM4Z, none (B); with pGEM4Z, 1 μM exogenous PQQ (C); with pSLY4, none (D); with pSLY4, 1 μM exogenous PQQ (E); with pGAB1d, none (F); with pGAB1, none (G); with pGAB1d, 1 μM exogenous PQQ (H); and with pGAB1, 1 μM exogenous PQQ (I). *, position of the gluconic acid peak.

scription-translation product of pSLY4. This ORF (designated *gabY*) codes for a polypeptide with a deduced molecular mass of 14.235 kDa, versus 14.75 kDa for the coupled in vitro transcription-translation product (Fig. 3). While other ORFs might exist in the unsequenced region of the pSLY4 insert, unidirectional deletion analysis data showed that this region was not necessary for gluconic acid production (as discussed below).

On the basis of these data, pSLY4 was deleted and subcloned by the strategy shown in Fig. 1. The resulting clone, pGAB1, retained the 396-base ORF downstream from the SP6 promoter as confirmed by sequence analysis. This plasmid was Mps^+ and produced the 14.75-kDa protein when analyzed by coupled transcription-translation (Fig. 3b). The insertion of a 4-bp addition at the unique *Xho*I site at position 138 of *gabY* via Klenow fragment repair resulted in the conversion of the base sequence from 5′-CTCGAG-3′ to 5′-CTCGATCGAG-3′, with the resultant insertion of 1 amino acid (AUC [i.e., I]) and a 1-base reading frame shift for the rest of the sequence. This reading frame shift would be expected to result in the reading of residues 162 to 164 of the original sequence as a UGA stop codon. The plasmid containing the Klenow fragment repair was designated pGAB1d. Figure 3b shows that the 14.7-kb translation product is no longer made by pGAB1d. Resolution of the gel was not good enough to identify the expected low-molecular-mass translation product. Figure 4F shows that JM109 carrying pGAB1d no longer produced detectable levels of gluconic acid in the absence of exogenous PQQ (see the next paragraph).

JM109 strains carrying pSLY4, pGAB1, pGAB1d, or pGEM4Z were analyzed for the ability to produce gluconic acid in glucose minimal medium. The results of these assays are shown in Fig. 4. As expected, JM109(pGEM4Z) produced gluconic acid only in the presence of 1 μM exogenous PQQ. Both JM109(pSLY4) and JM109(pGAB1) produced gluconic acid in the absence of PQQ, verifying the results of the plate assays. Surprisingly, the presence of PQQ in the medium produced an apparent synergistic interaction with the *P. cepacia* gene. The presence of both factors (1 μM exogenous PQQ and the *P. cepacia* gene on the plasmid) resulted in an approximate 10-fold enhancement in gluconic acid production compared with levels produced in the presence of either factor alone. JM109(pGAB1d) did not produce gluconic acid unless 1 μM exogenous PQQ was present in the medium.

Peak integration allowed us to approximate the amount of gluconic acid being produced by the various strains. JM109 (pSLY4) produced approximately 0.6 mM gluconic acid in the absence of exogenous PQQ. JM109 grown with 1 μM exogenous PQQ produced approximately 0.32 mM gluconic acid. JM109(pSLY4) plus 1 μM PQQ produced 4.5 mM gluconic acid. By comparison, *P. cepacia* E-37 produced between 3.0 and 5.0 mM gluconic acid when grown in 1% glucose minimal medium (17). Therefore, while a relatively small percentage of exogenous glucose is oxidized in the recombinant and/or PQQ-supplemented systems, the levels of activity appear to be physiologically reasonable.

The results of several control experiments are shown in Table 2. With HB101, the results are qualitatively similar to those seen with JM109. HB101 with exogenous PQQ produced 1.5 mM gluconic acid, while HB101(pGAB1) without PQQ produced 3.0 mM gluconic acid. The apparent synergistic interaction of pGAB1 and PQQ was seen with the same approximately 10-fold enhancement of exogenous gluconic acid [32 mM for HB101(pGAB1) with PQQ]. Only HB101(pGAB1) was capable of gluconic acid production in the absence of exogenous PQQ. AG121(pGAB1) did not produce gluconic acid. These data appear to demonstrate that pGAB1 acts via

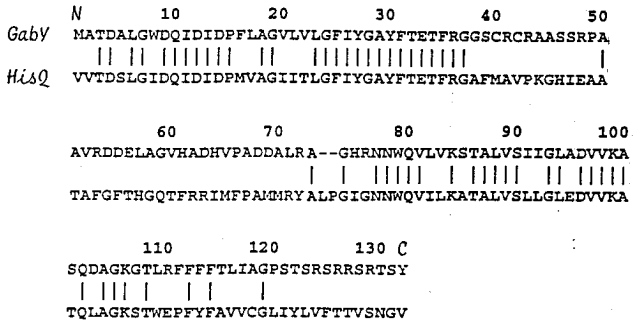

FIG. 5. Alignment of the amino acid sequences of GabY and a region of HisQ (total length, 228 amino acids) by using the FASDB algorithm of the Intelligenetics Suite computerized DNA sequence analysis software. The numbers above the sequences correspond to GabY position numbers. The first N-terminal region of high homology (vertical lines) corresponds to amino acids coded for by nucleotides 1,207 to 1,311 of the *hisQ* gene, as designated by the numbering system of Higgins et al. (11) for the histidine transport operon of *Salmonella typhimurium*. The second region of homology corresponds to nucleotides 1,420 to 1,555 of this same operon.

the quinoprotein GDH because of control experiments that demonstrate that AG121 is apoGDH$^-$. These controls are that (i) AG121 does not produce gluconic acid in the presence of exogenous PQQ and (ii) AG121(pGP478) produces gluconic acid only in the presence of PQQ.

The deduced amino acid sequence of *gabY* (i.e., GabY) has no apparent sequence homology to any previously identified direct oxidation pathway gene. Preliminary amino acid sequence analysis indicates that this gene does have two regions highly homologous to the membrane-bound components of the family of periplasmic permeases that include *hisQ* and *glnP*. A preliminary analysis of the homology to HisQ is shown in Fig. 5. On the basis of the model published by Kerppola and Ames (14), it appears that the N terminus of GabY corresponds to the first (i.e., N-terminal) periplasmic domain of HisQ as well as most of the third membrane spanner (QS3). There is no homology for the next 32 amino acids (amino acids 38 to 72 of GabY). It has been proposed that this region in HisQ contains the hydrophilic cytoplasmic domain that interacts with the conserved (ATP-binding) subunit of the transport system (11, 13). Hydropathicity analysis using the Hopp and Woods values (12) indicated that GabY is also relatively hydrophilic in this region. The second region of homology corresponds to the fourth membrane spanner and the second (i.e., C-terminal) periplasmic loop of HisQ. We note that the highly conserved EAA of the hydrophilic domain of the HisQ family of membrane-bound proteins (11) is missing from GabY. However, variation in this sequence is seen (e.g., EAG in GlnP). GabY does have an ELA sequence in its postulated cytoplasmic loop, although it is 5 amino acids further downstream from the first highly homologous region than the EAA of HisQ (Fig. 5). The implications of this homology are not clear at present, but it is reasonable to propose that GabY is a transmembrane protein with two periplasmic domains, two membrane spanners, and a cytoplasmic hydrophilic domain.

We were not able to obtain an absolute match between the DNA sequences of the coding and template strands of *gabY*. However, the first 500 bases of the coding strand downstream from the SP6 promoter of pSLY4 were independently sequenced three times. This sequence contains the region of GabY with the highest deduced homology to HisQ. Preliminary comparisons with published models for HisQ topology indicate that the deduced sequence of GabY diverges from HisQ almost precisely at the point of emergence from the

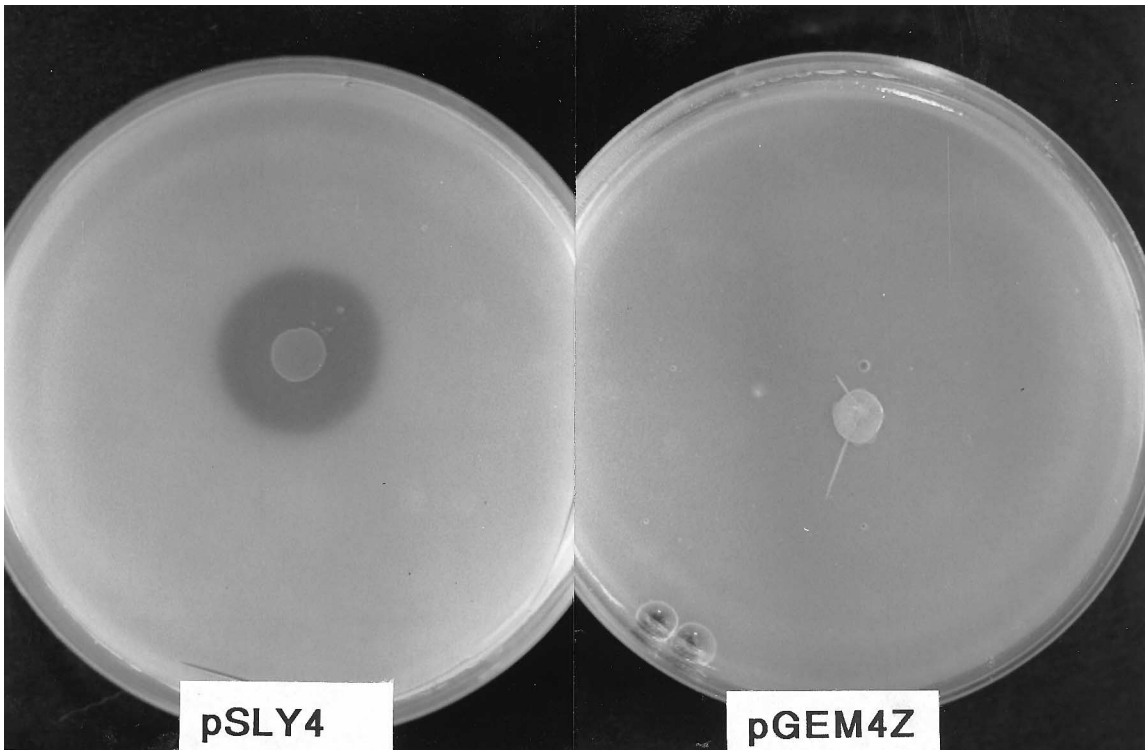

FIG. 6. Screening for Mps$^+$ phenotype. The presence of pSLY4 in *E. coli* JM109 results in strong expression of the Mps$^+$ phenotype, as visualized by the large clear zone around the 10-μl spot of overnight bacterial culture. This clear zone is where the hydroxyapatite in the medium has been solubilized. A pGEM4Z culture is shown for comparison.

membrane into the cytoplasmic space and realigns where these proteins reenter the membrane. This precise match in protein domains and transition regions argues strongly that the DNA sequence reported in Fig. 2 is generally correct.

The studies summarized in Table 2 clearly indicate that, in the presence of pGAB1, gluconic acid is produced only if the *E. coli* strain expresses a functional *gcd* gene. This genetic evidence does not address the question of whether the quinoprotein holoenzyme is actually formed. While it is generally believed that *E. coli* K-12 strains lack genes for PQQ biosynthesis, Biville et al. have reported the deblocking of cryptic *E. coli* PQQ synthase genes (2) and more recently have placed in GenBank an *E. coli* sequence able to complement *pqqE* and *pqqF* of *Methylobacterium organophilum* (accession number X71917). At present, we cannot exclude the possibility that GabY activates cryptic *E. coli* PQQ genes. Alternatively, GabY might catalyze the production of PQQ via an alternative biosynthetic pathway. Finally, expression of GabY might, in some way, result in production of an alternative redox cofactor that can be used by apo-GDH.

If, as predicted by homology with HisQ, GabY is a membrane-spanning protein with both periplasmic and cytoplasmic domains, theoretical arguments could be made for transport, signal transduction, or enzymatic function. However, any model would also have to explain the apparent synergistic interaction between the presence of pSLY4 or pGAB1 and 1 μM exogenous PQQ. If these two systems were independent, one would expect gluconic acid production in the presence of both factors to be the sum of the two systems acting alone. Alternatively, if GabY acted only to enhance PQQ binding to preexisting apo-GDH, there should be no effect of GabY alone. Homology to HisQ, a membrane-bound transport protein, does not preclude any of these possible roles. For example, the UhpC protein is similar in length, amino acid sequence, and transmembrane topology to the UhpT family of transport proteins but plays a regulatory role and is not a subunit of the transport system (13).

It is important to note that the DNA in pSLY4 was originally isolated as part of an effort to identify functional mineral phosphate-solubilizing genes in *P. cepacia*. As shown in Fig. 6, the presence of pSLY4 in *E. coli* results in a strong Mps$^+$ phenotype. On the basis of the evidence presented here, it is reasonable to propose that *gabY* plays some type of role in the expression and/or regulation of the direct oxidation pathway in *P. cepacia* and, therefore, may act as a functional Mps gene in vivo.

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